Isolation of *Bacillus circulans* and *Paenibacillus polymyxa* Strains Inhibitory to *Campylobacter jejuni* and Characterization of Associated Bacteriocins

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ABSTRACT

We evaluated anti-*Campylobacter* activity among 365 *Bacillus* and *Paenibacillus* isolates from poultry production environments. One novel antagonistic *Bacillus circulans* and three *Paenibacillus polymyxa* strains were identified and further studied. Cell-free ammonium sulfate precipitate (crude antimicrobial preparation) was obtained from each candidate culture. Zones of *Campylobacter* growth inhibition surrounding 10 μl of this crude antimicrobial preparation were quantified using a spot test. *Campylobacter* growth resumed when the preparation was preincubated with selected protease enzymes, demonstrating peptide characteristics consistent with a bacteriocin. These peptides were further purified using combinations of molecular mass resolution and ion exchange chromatography. Molecular masses of the peptides were estimated at approximately 3,500 Da by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Isoelectric focusing was used to determine the pI values of the peptides. Amino acid sequences of the bacteriocins and more precise molecular masses were obtained by matrix-assisted laser desorption and ionization–time of flight (MALDI-TOF) analysis. The bacteriocin from *P. polymyxa* NRRL B-30507 had a pI of 4.8, that from *P. polymyxa* NRRL B-30509 had a pI of 7.2, that from *P. polymyxa* NRRL B-30508 had a pI of 4.8, and that from *B. circulans* NRRL B-30644 had a pI of 7.8. The amino acid sequences were consistent with those of class IIa bacteriocins. These antagonists and the corresponding bacteriocins may be useful in the control of *Campylobacter* infection in poultry.

Normal intestinal bacteria are critical to the health of any host animal. The host derives benefit through the digestive metabolic processes mediated by the native bacterial biota. From the perspective of the intestinal bacteria, competition and consequent evolution provide nutrients and living space and increase reproductive potential, enabling certain strains and species to gain survival advantage. During bacterial evolution, bacteriocin production has occurred. Bacteriocins are antagonistic to other organisms within a given competitive niche and thus provide an ecological advantage. These bacteriocins are typically low-molecular-weight polypeptides and are classified based on differences in molecular weights *(13)*. These compounds can be digested easily into their component amino acids by host protease enzymes. Bacteriocins may represent a significant component of the benefits derived from competitive exclusion *(18)*.

Nurmi and Rantala *(18)* originally described the advantages of competitive exclusion in controlling *Salmonella* colonization among newly hatched chicks by using an undefined bacterial flora derived from the feces of healthy adult birds. This approach is an attractive alternative to current husbandry practices involving synthetic antibiotics. A

mucosal competitive exclusion flora was described *(24)* as an anaerobic subculture derived from the scrapings of the intestinal mucosal linings in healthy adult hens. This undefined flora provided excellent protection against *Salmonella* colonization in chickens but provided only inconsistent control of *Campylobacter* colonization *(23)*. Competitive exclusion occurs within the intestinal tract of wild birds and contributes to healthy gut ecology. However, the U.S. Food and Drug Administration has been reticent to approve commercial distribution of mucosal competitive exclusion flora within the United States primarily because of the undefined nature of these products. The application of a complex, uncharacterized competitive exclusion microflora may result in only short-term benefit, may provide inconsistent results, and may increase the potential for the development of antibiotic resistance *(7)*.

A significant reduction in broiler chicken colonization by *Campylobacter jejuni* through the application of carbohydrate supplements with *Citrobacter diversus (22)*, *Klebsiella pneumoniae (24)*, and *Escherichia coli (25)* has been reported *(20)*. There was also evidence of a significant decrease in *C. jejuni* concentration in intestinal samples from infected broiler chickens after treatment with poultry-isolated cultures of *Lactobacillus acidophilus* and *Streptococcus faecium (17)*. However, these treatments have not been applied within the commercial poultry industry.

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Diverse biological activities are common among *Bacillus* spp. *(1, 11, 14, 15)*, which may synthesize numerous amino acids as protein components of biologically active substances *(21, 22)*. With the exception of some isolates *(26)*, most members of the genus *Bacillus* are harmless to warm-blooded host animals and are phylogenetically related to lactobacilli *(6)*. Owing to these desirable characteristics, bacteria within the genus *Bacillus* have been used widely as probiotics and are used in Russia in both human medicine and veterinary practice *(21)*. Related *Paenibacillus* spp. are associated with nitrogen fixation and the rhizobia of plant roots and are facultative gram-positive anaerobic bacteria that form endospores.

Our study involved screening representative isolates of *Bacillus* and *Paenibacillus* for activity against *Campylobacter* to provide data on identification, purification, and characterization of substances responsible for antagonism. We isolated highly antagonistic cultures of *Bacillus* and *Paenibacillus* from the intestines of birds. The native components of characterized antagonists are low-molecular-weight peptides, i.e., bacteriocins, that have anti-*Campylobacter* activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Anti-*Campylobacter* activity of selected isolates of *Bacillus* and *Paenibacillus* was assessed under in vitro conditions. Target bacteria included four *C. jejuni* isolates from broiler chickens in Russia (B1, L4, F2, and K1) and strain NCTC 11168. All isolates were from the culture collection of the State Research Center for Applied Microbiology (SRCAM; Obolensk, Russia) and were collected as previously described *(25)*. Isolates of *C. jejuni* were grown on *Brucella* agar (SRCAM) or *Campylobacter* agar (HiMedia, Lab. Ltd., Mumbai, India) containing 5% partially lysed blood incubated at 42°C for 24 to 48 h under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) obtained by atmosphere replacement.

Bacillus and Paenibacillus isolates producing antimicrobial substances. *Bacillus* and *Paenibacillus* spp. were isolated from about 1.0 g of cecal, crop, or stomach contents of birds or from about 1.0 g of soil, suspended in 10 ml of sterile saline solution, and heated at 80°C for 15 min. Aliquots (0.1 ml) of 1:50 and 1:2,500 dilutions of these suspensions were spread plated onto either plate count agar (SRCAM) or starch agar (Difco, Becton Dickinson, Sparks, Md.). Plates were incubated at 30°C for 24 and 48 h. Characteristic bacillus morphology was confirmed by microscopy. Morphologically discrete colonies were selected and reseeded onto plate count agar or starch agar and incubated at 30°C for 48 h. Spore-forming cultures were preserved in vials containing starch agar. The morphology of the isolates matched the description of *Bacillus* and *Paenibacillus* spp. Candidate isolates were identified as *Bacillus* spp.–like organisms according to the API 50 CH typing system (bioMérieux, Marcy l'Etoile, France).

Spot test to characterize antagonism. Antagonistic activity against *Campylobacter* was evaluated by plating suspensions containing 10^8 CFU/ml of the candidate isolates onto starch agar and incubating at 30°C for 2 to 3 days until spores formed. Starch agar cubes (0.5 cm^3) were cut out and transferred onto *Brucella* agar or *Campylobacter* agar supplemented with 5 to 10% partially lysed blood, 10 μg/ml rifampicin, and 2.5 μg/ml polymyxin. These plates were previously seeded with suspensions of individ-

ual *C. jejuni* isolates. Plates were incubated at 42°C for 24 to 48 h under microaerobic conditions. Antagonistic activity was evaluated by measuring the diameter of the zones of *C. jejuni* inhibition surrounding the cubes containing the *Bacillus-Paenibacillus* cultures.

Production of crude antimicrobial preparations. Antagonists were grown in 250 ml of modified Kugler's broth medium *(14)* supplemented with alanine, tryptophan, and glucose at 32°C for 40 h under aerobic conditions. The stationary cultures were centrifuged at 2,500 × *g* for 10 min to remove most of the viable cells. The decanted supernatant was mixed with 80% saturated ammonium sulfate and incubated at 4°C for 24 h to precipitate protein compounds. Following centrifugation at 10,000 × *g* for 20 min, the sediment was resuspended in 1.5 ml of 10 mM phosphate-buffered saline (PBS; pH 7.0) and dialyzed overnight against 2.5 liters of the same buffer. The solution obtained was designated the crude antimicrobial preparation (CAP). Each CAP was filtered through a 0.22-μm-pore filter (Millipore, Bedford, Mass.).

Purification of bacteriocins. Bacteriocins were purified from the CAP by gel filtration and ion exchange chromatography. The CAP was placed onto a Superose 12HR 16/50 column (1.6 by 50 cm; Pharmacia, Uppsala, Sweeden) treated with 50 ml of PBS (pH 5.8). Bacteriocins were eluted with the same buffer at 0.85 ml/min. Eluted fractions were tested for their activity against three *C. jejuni* strains by employing the spot test with the eluted fractions. The concentration of the protein was measured as previously described *(16)*. Further purification of fractions with antimicrobial activity was performed on a Mono Q HR 5/5 column (1.5 by 20 cm; Pharmacia) or a CM-Sepharose column (1.5 by 20 cm; Pharmacia). The Mono Q column was treated with 20 ml of PBS (pH 7.8) at 5 ml/min. Bacteriocins were eluted with the same buffer at NaCl gradients of of 0.1, 0.15, 0.3, and 0.5% and a flow rate of 1.5 ml/min. The CM-Sepharose column was treated with 75 ml of PBS (pH 5.8) at a flow rate of 5 ml/min. Bacteriocins were eluted with 50 ml of NaCl buffer gradients from 0.4 to 1.2% at a flow rate of 2 ml/min. Antimicrobial activity and concentration of proteins were determined for each fraction.

Measuring CAPs and bacteriocins. One milliliter of sterile CAP containing the bacteriocins was diluted in 1 ml of PBS (pH 7.0) and deposited into a well within the agar plates (blood-supplemented *Campylobacter* agar and plate count agar) previously seeded with cells of the target bacteria. Cultures of *C. jejuni* were grown at 42°C under microaerobic conditions. After 24 h of incubation, zones of growth inhibition of the target bacteria surrounding the CAP and bacteriocins were noted. Activity of the CAP and bacteriocin was measured in arbitrary units (AU) per milliliter of the preparation and defined as a maximal dilution at which a visible zone of inhibition surrounded the sample well *(9)*. All the experiments were conducted in duplicate.

Characterizing CAPs and bacteriocins by electrophoresis. CAPs and bacteriocins were separated by socium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) in a continuous 16.5% agarose gel (9 by 12 cm) with 1% SDS in Tris-glycine buffer (pH 8.3). To determine molecular weights of protein fractions, strips were stained with a solution containing 0.25% Coomassie brilliant blue G-250, 50% ethanol, and 7% acetic acid. After electrophoresis at 100 mA for 4 h, each lane containing the preparation was cut. The strips were tested against three target *C. jejuni* isolates and *C. jejuni* strain NCTC 11168 by the method of Bhunia et al. *(3)*. Strips were immersed in a solution of 15% ethanol and 8% acetic acid for 1.5 h, washed twice with distilled

TABLE 1. *Inhibitory activity of selected* Bacillus *and* Paenibacillus *isolates against test strains of* Campylobacter jejuni *(NCTC 11168, B1, L4, F2, and K1)*

Antagonist identification	Source of strain	Inhibition zone diameter (mm)					Hemolytic activity[a]
		NCTC 11168	B1	L4	F2	K1	
37	Broiler chicken, crop	5	3	4	5	4	−
114	Broiler chicken, intestine	6	5	5	5	5	−
119	Broiler chicken, intestine	4	3	5	5	6	−
236	Broiler chicken, intestine	2	2	3	3	4	±
265	Broiler chicken, intestine	2	2	2	4	6	+
346	Broiler chicken, crop	3	5	3	3	6	±
358	Broiler chicken, crop	3	3	3	4	5	+
361	Broiler chicken, crop	3	3	3	2	4	+
362	Broiler chicken, crop	4	4	4	3	5	+
397	Broiler chicken, intestine	4	5	3	4	6	+
442	Quail, intestine	4	3	4	4	4	+
462	Quail, intestine	3	5	4	4	5	+
538	Broiler chicken, crop	4	5	5	4	5	+
592	Broiler chicken, crop	5	5	4	4	5	±
602	Broiler chicken, crop	6	5	5	5	6	−

[a] +, presence of activity; ±, weak activity; −, absence of activity.

water for 4 h, placed into sterile petri plates, and overlaid with either with soft *Campylobacter* agar (1.2% agar) supplemented with 5% lysed blood or semisolid plate count agar seeded with target bacteria. *C. jejuni* was incubated at 42°C for 48 h under microaerobic conditions. After incubation, zones of inhibition were measured.

Characterizing CAPs and bacteriocins by isoelectrofocusing. Samples of CAPs and bacteriocins were placed onto isoelectrofocusing gels (pH 4.4 to 10.0; Novex, San Diego, Calif.) and exposed to isoelectrofocusing (100 V for 1 h, 200 V for 2 h, and 500 V for 30 min) in an XCell II Mini-Cell (Novex). After isoelectrofocusing, the gel was washed for 30 s with distilled water without fixation. Each gel strip was cut out and stained with Coomassie blue R-250 to determine pI values of the various separated protein fractions. Additional strips were evaluated for inhibitory activity of protein fractions against the above strains using previously described methods *(27)*.

Effect of enzymes, temperature, and pH on antimicrobial activity of bacteriocins. A small amount (10 μl) of each of the following enzymes (Sigma, St. Louis, Mo.) was transferred to tubes containing 2.0 mg/ml of bacteriocins: chymotrypsin (100 mg/ml; >40 U/mg), protease (200 mg/ml; 5 U/mg), papain (60 mg/ml; approximately 3 U/mg), lysozyme (750 mg/ml; 50,000 U/mg), and lipase (100 mg/ml; 2,500 U/mg). After 3 h of incubation at 37°C, the mixture of bacteriocins and enzymes was analyzed for antimicrobial activity using the spot test. Untreated bacteriocin served as a positive control.

To study thermostability of the bacteriocins, the samples (2 mg/ml) were boiled in a water bath for 15 min and then cooled, and their antimicrobial activity was assessed.

Bacteriocin preparations also were used to evaluate the effect of pH on biological activity. Drops of 10 mM NaOH or 10 mM HCl were added to 2 mg/ml bacteriocin preparations to assess sample stability at pH values of 3 to 10. Samples that were pH adjusted were incubated at 37°C for 2 and 24 h and at 90°C for 20 min. Samples were then adjusted to pH 7.2 by addition of 4 mM sterile phosphate buffer and analyzed for antimicrobial activity.

Amino acid sequencing. Amino acid sequences were determined by Edman degradation with a 491 cLC Automatic Sequencer (Applied Biosystems, La Jolla, Calif.) as per the manufacturer's instructions.

Mass spectrometry. Determination of the molecular mass of bacteriocins from *P. polymyxa* B-30507 and B-30509 and *B. circulans* B-30644 was performed by matrix-assisted laser desorption and ionization–time of flight mass spectrometry (MALDI-TOF MS) with an electrospray-ionizing mass spectrometer (API III TAGA 6000E, CJEX, Mumbai, India) as per the manufacturer's instructions.

RESULTS

***Bacillus* and *Paenibacillus* isolates producing antimicrobial substances.** Antagonistic activity against *C. jejuni* was evaluated using the spot test for 365 isolates originally screened as *Bacillus* spp. Fifty-six of these isolates exhibited antagonism to *C. jejuni,* and 15 of the most antagonistic isolates were chosen for further research (Table 1). Isolates SRCAM 37 (NRRL B-30507), 114, 119 (NRRL B-30508), 592, 602 (NRRL B-30509), and 1580 (NRRL B-30644) manifested antagonistic activity and differed from one another in their growth characteristics and ability to cause lysis of erythrocytes. These six strains were identified as *Bacillus* spp.–like organisms. *B. circulans* (NRRL B-30644) and three *P. polymyxa* (NRRL B-30507, B-30508 and NRRL B-30509) isolates were identified and deposited under provisions of the Budapest Treaty.

Biochemical characterization and antimicrobial activity of CAPs and bacteriocins. Table 2 provides data on the antagonistic activity of CAPs against isolates of *C. jejuni.* The CAPs of SRCAM isolates 37, 114, 119, 592, 602, and 1580 were most effective against the target isolates of *Campylobacter.* The CAPs from these isolates manifested spot test titers of *C. jejuni* inhibition ranging from 1:3,200 to 1:25,600 AU/ml.

TABLE 2. *Anti-*Campylobacter jejuni *activity of crude antimicrobial preparations (CAPs) derived from isolates of* Bacillus *and* Paenibacillus *and evaluated by a spot test*

C. jejuni strains	CAP activity (AU/ml)[a]													
	37	114	119	236	265	346	361	397	442	462	538	592	062	1580
NCTC 11168	12,800	12,800	6,400	3,200	1,600	3,200	800	800	800	1,600	3,200	3,200	6,400	12,800
F-2	12,800	12,800	6,400	1,600	1,600	3,200	800	400	400	1,600	1,600	6,400	6,400	12,800
L-4	12,800	12,800	6,400	3,200	1,600	800	400	400	800	400	1,600	3,200	6,400	12,800
B-1	12,800	12,800	6,400	3,200	1,600	800	200	200	200	400	3,200	6,400	6,4000	12,800

[a] Arbitrary units (AU) of activity per milliliter of the preparation is defined as the maximum dilution at which a clearly visible zone of *Campylobacter* inhibition surrounded the sample well.

SDS-PAGE analysis revealed only one protein fraction (approximately 3.5 kDa) that produced a clear zone of growth inhibition against *C. jejuni* (Fig. 1).

Isoelectrofocusing allowed us to distinguish four distinct protein fractions from the CAPs at different pI values. Anti-*C. jejuni* activity was observed at pI 4.8 for isolate SRCAM 37 (Fig. 2), at pI 7.2 for SRCAM 602, and at pI 7.8 for SRCAM 1580 (Fig. 3).

Production of purified bacteriocins. Based on the pI and molecular weights of the bacteriocins, we applied both gel filtration and ion-exchange chromatography for their further evaluation. Because the anti-*Campylobacter* active fractions present in preparations SRCAM 37, 114, 119, 592, 602, and 1580 had similar molecular weights, use of

Superose 12HR gel filtration was selected as the first step in further purification of the CAPs. Fractions generating peaks at $\lambda = 280$ nm were analyzed for their antagonistic activity against *C. jejuni* using both a spot test and SDS-PAGE (Fig. 1).

Ion-exchange chromatography was then used to further
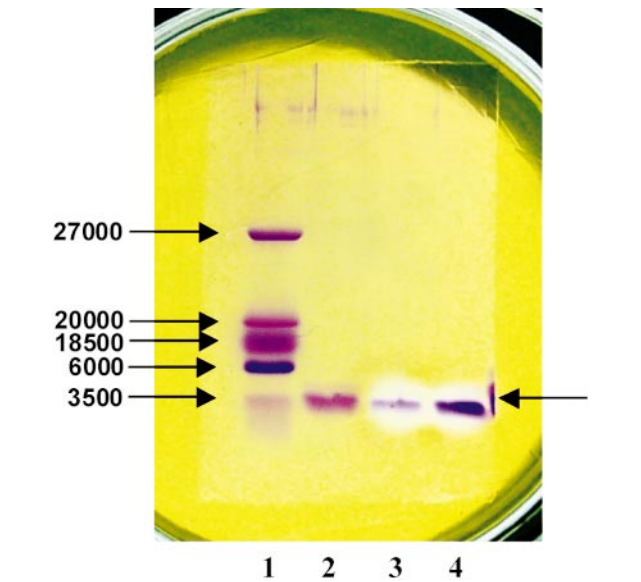
FIGURE 1. *Direct demonstration of* Campylobacter jejuni *inhibition by bacteriocins 37 and 602 produced by* Paenibacillus polymyxa *NRRL B-30507 and NRRL B-30509, respectively. The SDS polyacrylamide gel was overlaid with* C. jejuni *(ATCC 11168) contained in 1.2%* Brucella *agar to determine which secreted protein band(s) corresponded to antimicrobial activity and a specific molecular mass. Lane 1, molecular mass markers (Pharmacia) consisting of 27,000, 20,000, 18,500, 6,000, and 3,500 Da; lane 2, purified insulin; lane 3, purified bacteriocin 602; lane 4, purified bacteriocin 37. The protein band around which growth inhibition was observed corresponded to a mass of about 3,500 Da (arrow).*
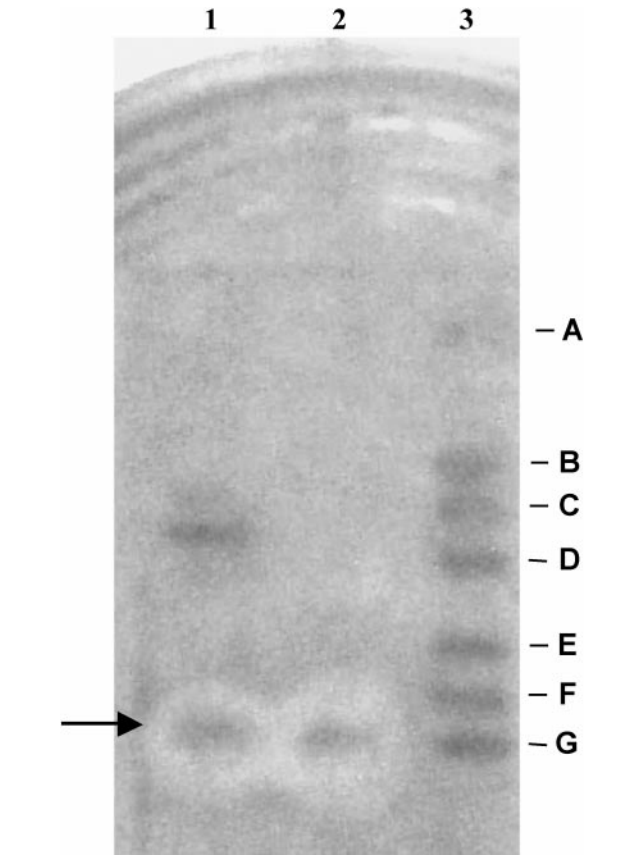
FIGURE 2. *Direct detection of bacteriocin 37 produced by* Paenibacillus polymyxa *NRRL B-30507 after isoelectrofocusing. The gel was overlaid with* Campylobacter jejuni *NCTC 11168 contained in 1.2%* Brucella *agar to determine which band(s) corresponded to the antimicrobial activity and isoelectric point. The bands in lane 1 (CAP 37) and lane 2 (purified bacteriocin 37) demonstrated antimicrobial activity, and those zones of growth inhibition (arrow) had a pI of about 4.8. Other bands did not demonstrate antimicrobial activity. Lane 3 contained pI standards (Protein Test Mixture 7, PI Marker Proteins, Serva Electrophoresis, Heidelburg, Germany): A, 8.45; B, 7.9; C, 7.5; D, 7.1; E, 6.3; F, 5.1; G, 4.7.*
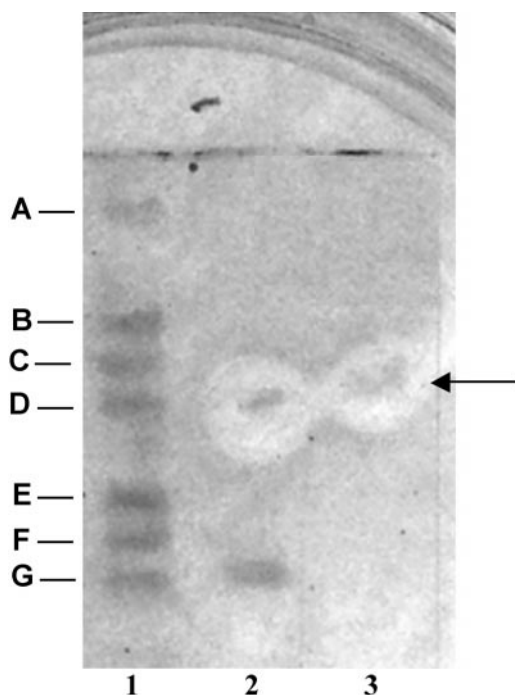
FIGURE 3. *Direct detection of bacteriocin 602 produced by* Paenibacillus polymyxa *NRRL B-30509 after isoelectrofocusing. The gel was overlaid with* Campylobacter jejuni *NCTC 11168 contained in 1.2%* Brucella *agar to determine which band(s) corresponded to the antimicrobial activity and isoelectric point. The bands in lane 1 were pI standards: A, 8.45; B, 7.9; C, 7.5; D, 7.1; E, 6.3; F, 5.1; G, 4.7. The bands in lane 2 (CAP 602) and lane 3 (purified bacteriocin 602) demonstrated antimicrobial activity, and those zones of growth inhibition (arrow) had a pI of about 7.2. Other bands did not demonstrate antimicrobial activity.*

purify and prepare quantities of the active fractions. Mono Q anion-exchange chromatography was used for preparations SRCAM 37, 114, 119, and 592 because isoeletrofocusing demonstrated an active fraction at pI 4.8 (Fig. 2). Chromatographic purification was performed by elution of active fractions with a single peak at $\lambda = 210$ nm in the presence of 0.3 M NaCl. Cation-exchange chromatography was applied to purify fractions from SRCAM 602 and 1580, which had active peptides at pI 7.2 and 7.8 (Fig. 3 for bacteriocin 602). After molecular sieving, the purified preparations from SRCAM 602 and 1580 were further refined by carboxymethyl (CM) cation-exchange chromatography. The active fraction was eluted as a single peak at $\lambda = 280$ nm in the presence of 0.8 M NaCl. Purity of the bacteriocins was obtained by Mono Q and CM chromato-

graphic separation methods. Results from the stepwise purification of the bacteriocin produced by NRRL B-30509 are presented in Table 3. The purified peptides were designated as bacteriocins, and their anti-*Campylobacter* bacteriocidal activity was identical to that of the CAPs.

Sensitivity of bacteriocins to enzymes, pH, and temperature. The bacteriocins lost their antimicrobial activity after being treated with β-chymotrypsin, proteinase K, and papain but retained activity when treated with lysozyme or lipase or being heated to 100°C for 15 min. The bacteriocins were stable at pH 3.0 to 9.0 but were inactivated at pH 10.

Amino acid sequences. Table 4 provides the amino acid sequences for the isolated and purified bacteriocins produced and secreted by *P. polymyxa* NRRL B-30507 and NRRL B-30509 and *B. circulans* NRRL B-30644. The conserved amino acid sequence of class IIa bacteriocins is also provided.

Mass spectrometry. Molecular masses of bacteriocins from SRCAM 37, 602, and 1580 (NRRL B-30507, B-30509, and B-30644) were confirmed by MALDI-TOF MS (Table 4).

DISCUSSION

We entered into this study without knowledge or expectations related to the mechanism of *Campylobacter* inhibition. We first searched for bacteria antagonistic to *C. jejuni,* intending to explain any inhibitory phenomenon. Using methods appropriate for the isolation of *Bacillus* and *Paenibacillus* spp., we were able to select 56 strains for further studies. We first attempted to use these isolates for competitive exclusion experiments in chickens, but we were not able to obtain satisfactory *Campylobacter* control for animal trials (data not shown). Consequently, we wanted to determine why in vitro results appeared promising but in vivo results were disappointing.

The antimicrobial properties involved in the reported zones of inhibition (Table 1) were of a proteinaceous nature, which we determined after demonstrating by the spot test that anti-*Campylobacter* activity was retained in the ammonium sulfate precipitate (Table 2). Subsequently, we used biochemical techniques of molecular weight sieving and isoelectrofocusing to further determine the proteinaceous nature of these moieties. These purified substances lost antimicrobial capacity after exposure to proteolytic enzymes. We also analyzed the amino acid sequences to ver-

TABLE 3. *Biochemical purification of bacteriocin 37 derived from* Paenibacillus polymyxa *(NRRL B-30507)*

Sample	Volume (ml)	Protein (mg/ml)	Specific activity (AU/mg protein)[a]	Purity (%)
Culture supernatant	150	1.5	17,066	0
CAP (centrifugation with $(NH_4)_2 SO_4$)	8.9	0.9	28,444	9.09
Superose-12 gel filtration	4	0.3	51,200	80.5
Mono Q anion-exchange chromatography	1.8	0.19	134,736	98.8

[a] Arbitrary units (AU) of activity per milliliter of the preparation is defined as the maximum dilution at which a clearly visible zone of *Campylobacter* inhibition surrounded the sample well.

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